INDUCTION OF NEW HOST-CODED PROTEINS IN PINUS ELLIOTTII SEEDLINGS IN RESPONSE TO PATHOGEN AND WATER STRESS

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Abstract.-A 55 kD stress protein was induced in tissue culture grown slash pine seedlings infected with conidial suspensions of pitch canker fungus. Induction was evident in both hypocotyl and root sections of the seedlings after a lag phase of 16 hours. The stress protein is best extracted from stressed tissues with slightly alkaline Tris-HCI (pH 8.65) buffer. We have also investigated protein synthesis in water-stressed slash pipe seedlings. Mannitol-induced water stress inhibited overall protein synthesis, with induction of unique stress proteins in the hypocotyl tissue. At -1.8 MPa a 46 kD stress protein was induced. At -2.5 MPa at least five stress proteins 82 kD, 71 kD, 70 kD, 64 kD and 58 kD were detected by SDS-PAGE and Fluorography.

Additional key words: Pinus elliottii, Hypocotyl, Stress protein, Fusarium moniliforme var. subglutinans, Water stress, SDS-PAGE, Protein induction.

The resistance of pine trees to pathogen and water stress is of large economic importance. The development of techniques to screen new germlines for stress tolerance has significant importance towards better understanding of the biochemistry of stress in trees as -well as the development of more resistant varieties. Higher plants under stress have evolved a variety of morphological and physiological mechanisms to cope with adverse biotic and abiotic stresses. The initial biochemical events which occur in plants exposed to pathogen and water stress appear to be quantitative and qualitative changes in protein metabolism. It has been proposed that the initiation of resistance or tolerance mechanisms is mediated by de novo gene activation resulting in the synthesis of new proteins which possibly have a role in plant protection against stresses (Antoniw and White 1983).

Pathogen stress: During the last few years, several groups have initiated studies on the response of plants to pathogen stress with emphasis on the analysis of gene expression (Van Loon 1985). Since genetic information is ultimately expressed in nucleic acids and proteins via the processes of transcription and translation, specific alterations in nucleic acid and protein metabolism are important aspects of pathogenesis. Transcriptional metabolism and regulation of pathogen stress induced proteins have not been studied in commercial woody plants, such as conifers.

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We now present experimental evidence indicating *de novo* synthesis of a new protein in slash pine (*Pinus elliottii)* in response to stress induced by the pitch canker fungus (*Fusarium moniliforme var.subglutinans).* Pitch canker incited by this fungus is a serious disease of pine plantations and seed orchards in the southern United States. The destructive potential of pitch canker is sufficient to warrant increased attention to identify cellular components of the host which may have a role in disease resistance.

Water stress: Pines exposed to water stress environment encounter several basic problems: (1) a reduction in the water potential of the sorrounding medium; (2) the buildup and interference of toxic metabolites with the normal biochemical and physiological processes of the cells; and (3) required nutrition ions must still be obtained despite the predominance of other ions. In stressful environments, higher plants show a variety of morphological and biochemical modifications. When plants undergo frequent periods of drought their ability to synthesize proteins is reduced (Bewley et al. 1983). if drought is not too severe, protein synthesis can resume upon subsequent return of the plant to full hydration. As a stress response, the effect of drought stress appears analogous to the heat shock response in animals, plants and bacteria. The stress treatment mediates a repression of pre-existing (non-stress) protein synthesis while inducing transcription and translation of a small number stress proteins (Dasgupta and Bewley 1984).

METHODS

We have established a tissue culture system to study the changes in protein synthesis in slash pine in response to pitch canker fungal infection and mannitol-induced water stress. This *in vitro* system with *Pinus elliottii* seedlings grown from embryos is used to study the induction of stress proteins at various time periods.

Establishment of seedlings: Slash pine seed were supplied by the Texas Forest Service. Seeds were placed in 30% hydrogen peroxide for 30 min and then soaked in 1% H_2O_2 at room temperature for 4 days. They were then surface sterilized in 15% Chlorox for 15 minutes and rinsed three times with sterile water. Embryos were removed aseptically, placed in Murashige -Skoog (MS) medium in sterile culture tubes, and grown in a controlled environment chamber with temperature of 24°C and 16 hr photoperiod (Murashige and Skoog 1962).

Fungal inoculum preparation and inoculation: The procedures for preparation of macro - and microconidia of *F. moniliforme* were modified from Barrows-Broaddus and Dwinnel. The initial fungal isolate was recovered from *Pinus elliottii var.elliottii* in Florida and was donated by G.M. Blakeslee (School of Forest Resources, University of Florida, Gainesville). Fungal cultures were grown on a PDA medium for 10 days with a 12 hr photoperiod at 24°C. Conidia were washed from the culture plates with sterile deionized water, and the number of conidia were estimated with a hemacytometer and the concentration was adjusted to 10^6 ml -1 . Ten ul of the inoculum were placed directly

on the hypocotyl with wounding by hypodermic needle. Controls were treated identically with distilled water in lieu of the inoculum. The plant material was incubated for 2, 4, 8, and 16 hr in an environmental chamber.

Mannitol-Induced Water stress: In separate set of experiments mannitol was added to the culture medium to obtain varying water potentials. Addition of 10% and 15% mannitol resulted in water potential of -1.8 MPa and -2.5 MPa respectively. In our preliminary studies on 'S-methionine incorporation into slash pine hypocotyls, we have eliminated the use of polyethylene glycol (PEG) as a suitable osmoticum for water stress. The PEG interferes with protein extraction and migration on gel electrophoresis. Mannitol (10% and 15%) have shown to elicit water stress symptoms and has no effect on migration of proteins on gel electrophoresis. A wescor (Logan, Utah) model HR33T hygrometer with model G52 thermocouple psychrometer chamber in the dew point mode was used to measure osmotic potential of the medium. Seedlings were water stressed for 2, 4, 8 and 16 hours.

In vivo labelling and extraction of proteins: At the end of each stress period the seedlings were excised aseptically and divided to cotyledons, hypocotyls, and root sections which were then incubated in 1 ml of a solution which contained 50uCi ml^{-1} of ^{35}S methionine (> 500 Ci/mol; ICN) for 2 hours. After labelling, the sections were removed and washed with a solution containing 1mM methionine. They were homogenized in ice-cold 50mM Tris-HCI buffer (pH 8.65), containing 20mM KC1, and 10mM $MgC1_2$. The slurry was centrifuged at 13,000g for 15 minutes, and the supernatant fraction was used for protein analysis. For measurement of protein synthesis, 200/11 of the supernatant was precipitated with 1 ml of 20% (w/v) trichloroacetic acid overnight in the cold. Fifty p.1 of the supernatant fraction were counted for total incorporation in 3 ml of a toluene scintillation mixture. The remaining supernatant and the pellet were washed with 5% TCA through fiberglass filters (Gelman Sciences Inc, A/E 2.5 cm diameter). The proteins trapped on the filter paper were rinsed twice with 5 ml of 95% ethanol and acetone. The filters were dried at 60°C and the radioactivity on the filters was directly counted in 3 ml of a toluene scintillation mixture.

Polyacrylamide gel electrophoresis: Total protein was estimated by the Bradford method using a Dynatech MR 650 microtiter reader (Bradford 1976). Gels were loaded based on equal amount of TCA precipitable counts from control and stress tissues. SDS-PAGE was carried out on 12% polyacrylamide gels containing 0.1% SDS (Laemmli 1970). After electrophoresis gels were treated with EN^3HANCE (New England Nuclear) prior to drying. Fluorography was performed on dried gels using Kodak X-Omat AR film at -70° C.

RESULTS AND DISCUSSION

Sixteen hours after inoculation, a new protein band was detected in extracts of both hypocotyl and root tissue from stressed tissue, but not in the uninoculated control tissues (Fig 1). The protein found in stressed pine hypocotyl and root tissue is of relatively high molecular weight (55 kD).

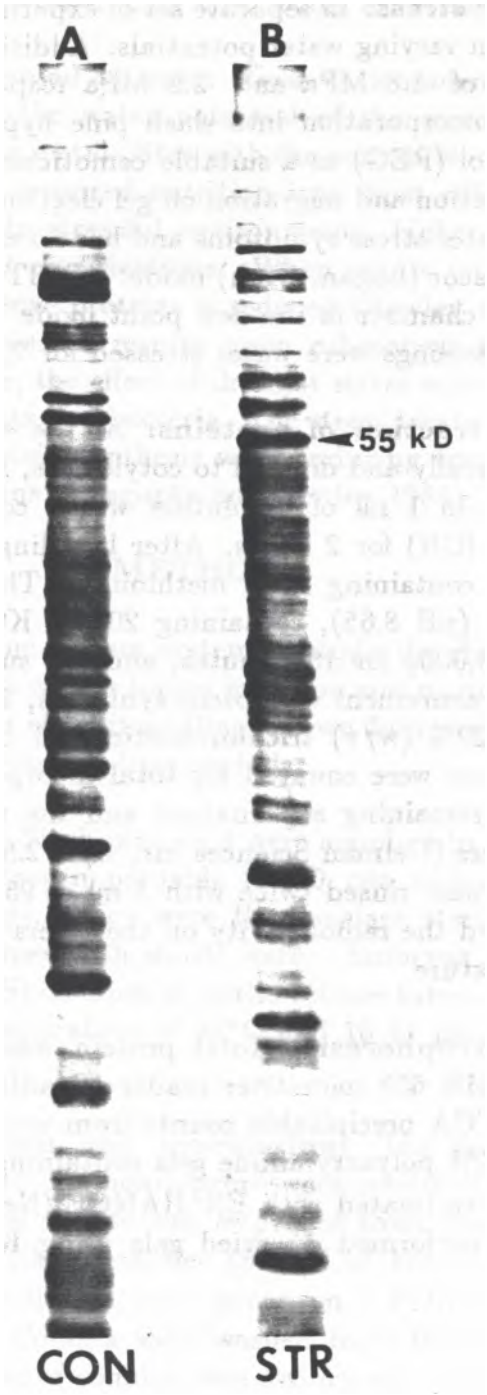

Figure 1. SDS-Polyacrylamide gel pattern showing induction of a 55 kD stress protein in response to pitch canker fungal infection (lane B). Lane A is the control tissue. Each lane contains equal amount of radioactive counts (2x 10^5 cpm).

Relative mobilities were calculated for each of the standard proteins and for the stress protein. The apparent molecular weight of the stress protein was determined by matching the R_f value with the appropiate point on the standard curve. Analysis of 2, 4, and 8 hr stressed tissue did not reveal any synthesis of new proteins. Under these incubation times there was no fungal growth on the stressed tissue. In order to determine if the observed, high-molecular weight protein was a result of *de novo* synthesis in host tissue and not of fungal origin, native proteins in the fungal inoculum were analysed by SDS-PAGE, and stained with Bio-Rad's silver stain. No proteins were detected in the inoculum, suggesting that the new protein detected is of host origin and induced in response to pathogen stress. We hypothesize that the high molecular weight stress-protein induced in the pine hypocotyls is as a result of chemical mediation eliciting a signal for gene activation. Although the induction of stress protein was not detected until 16 hours after fungal challenge, Bradford assay showed increase in total protein in the stressed tissue, when compared to the uninoculated controls.

A striking feature of pathogen stress response in slash pine seedlings is that the stress protein synthesis occurs in addition to the normal protein synthesis. This is very different from the drought stress response of slash pine seedlings in which normal protein synthesis decreases so that only drought stress proteins are detectable on a gel. Both qualitative and quantitative changes were detected in protein patterns in slash pine hypocotyls subjected to -1.8 MPa and -2.5 MPa water stress for 24 hr. At -1.8 MPa slash pine hypocotyls responded to stress by induction of a 46 kD stress protein. At -2.5 MPa at least five stress proteins 82 kD, 71 kD, 70 kD, 64 kD and 58 kD were detected by SDS-PAGE and Fluorography (Fig 2). The synthesis of several proteins was reduced by stress, although that of others intensified. Stress recovery experiments were done by transferring the stressed tissue onto the control medium. Protein induction in tissue stressed at -1.8 MPa for 24 hr appears to recover, whereas in tissue stressed at -2.5 MPa the overall protein recovery does not occur. While new proteins were induced in stressed tissues, their capacity to conduct *in vivo* protein synthesis when the tissues are returned to control media is low. We regard such tissues as having acutely stressed.

Some studies have documented that the drought stress inhibited the incorporation of amino acids into proteins. The protein synthesis data obtained in our study reveal good incorporation of ^{35}S met hionine label in the control tissue and a decreased incorporation in the stressed tissue. Water stress is a signal for the induction of new proteins, and we know at mild to moderate water stress the stress protein synthesis will eventually return to normal if that signal is removed. How the signal is percieved by the cell is a question that remains largely unanswered. We believe that under short term drought stress shock proteins are produced which are transient in nature and disappear when the stress is removed. However, in tissue stressed at -2.5 Mpa different pattern of protein synthesis occurs which includes strong induction of new proteins which are stable even after the stress is removed.

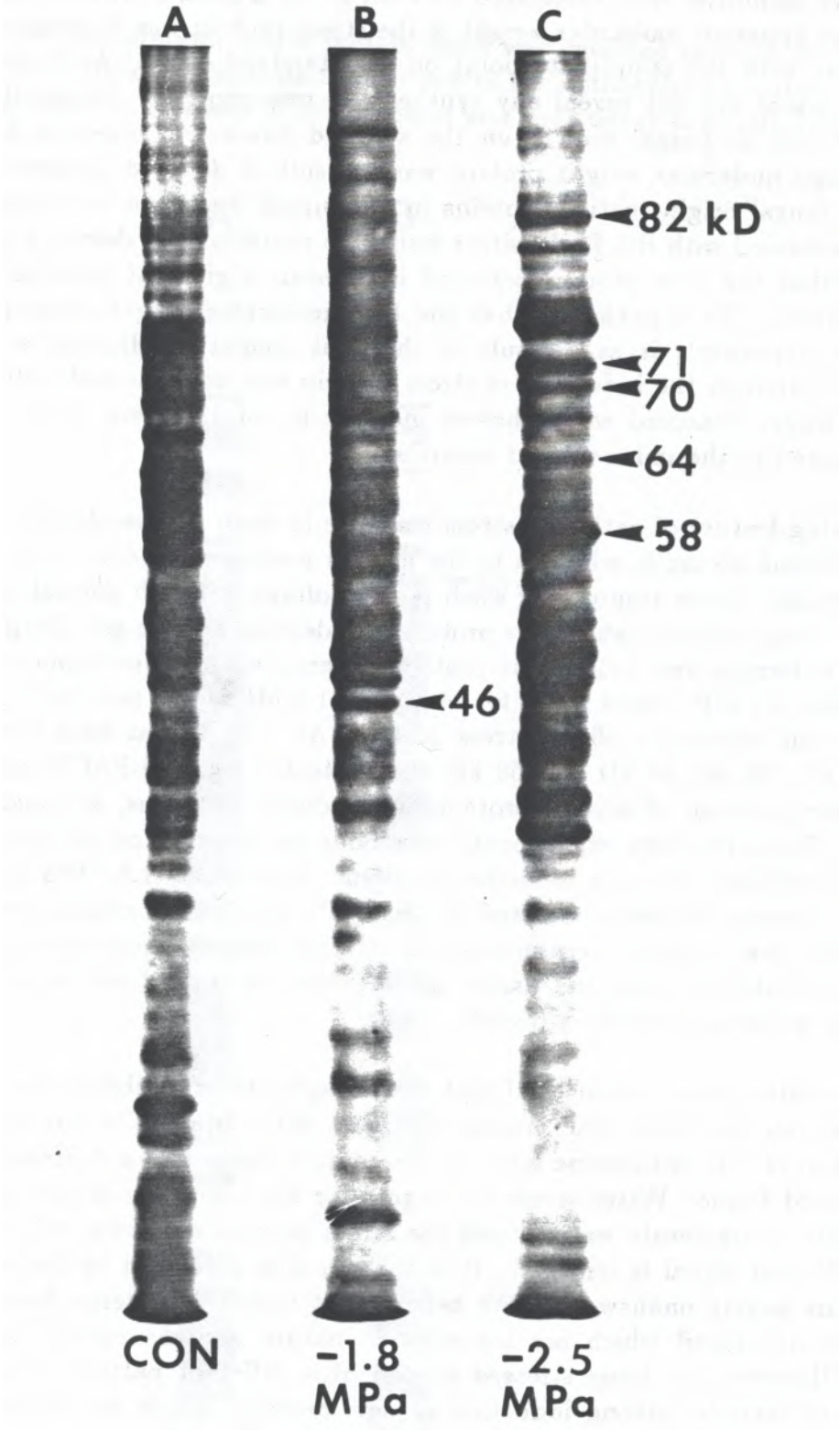

Figure 2. SDS-Polyacrylamide gel pattern showing induction of unique proteins in hypocotyl tissue in response to water-stress at -1.8 MPa (lane B) and -2.5 MPa (lane C). Lane A is the control tissue. Each lane contains equal amount of radioactive counts (2×10^6 cpm).

CONCLUSIONS

In general, stress proteins are absent in healthy growing plants. Induction of these proteins occurs in response to both biotic and abiotic stress conditions. While some researchers have suggested that the pathogen-stress proteins may have a role in induced systemic resistance, their function is still speculative. The evidence gathered thus far establishing a link between pathogen-stress proteins and induced resistance is comprehensive, but it is entirely correlative.

The resistance of pine trees to water stress has received much attention due its economic importance. Any metabolic adaptation to water stress involving a change in gene expression requires an alteration in protein and /or nucleic acids which may have a role in drought tolerance mechanisms. Cellular-level tolerance might be most amenable to genetic manipulation because few genes for metabolic processes are involved in osmoregulation. Additional advantage would include evaluating potential germplasm for drought tolerance, assuming that similarity exists between cellular level responses and whole-plant level responses under field conditions.

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